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**ANALYTICAL AND DIAGNOSTIC VALIDATION OF A FLOW CYTOMETRIC STRATEGY TO QUANTIFY BLOOD  
AND MARROW INFILTRATION IN DOGS WITH LARGE B-CELL LYMPHOMA**

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**Running headline:** canine large B-cell lymphoma staging by flow cytometry

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## Abstract

**Background** Lymph node (LN), peripheral blood (PB) and bone marrow (BM) samples are commonly analyzed by flow cytometry (FC) for the immunophenotyping and staging of canine lymphomas. A prognostic value for flow cytometric BM infiltration in dogs with large B-cell lymphoma (LBCL) was demonstrated. Aim of the present study was to define the analytical performances of this technique, and to establish a cutoff suitable to safely discriminate between infiltrated and non-infiltrated PB and BM samples.

**Methods** Large B-cells were added to control PB and BM samples, to achieve twelve different large B-cells concentrations, ranging from 0% to 50%. The percentage of large B-cells was recorded for each dilution, using a BD Accuri C6 flow cytometer. Accuracy was evaluated by Passing-Bablok regression analysis. Intra-assay precision was assessed at 0%, 1%, 3% and 10% dilutions evaluating the CVs of ten repeated acquisitions. ROC curves were drawn to identify the cutoffs most suitable to discriminate between 25 infiltrated (PARR-positive) and 25 non-infiltrated (PARR-negative) PB and BM samples, respectively.

**Results** Optimal analytical accuracy and precision were achieved. Almost all CVs were <10%. Negative controls had up to 0.5% large B-cells, with 50% and 22% CV in PB and BM samples, respectively. 0.56% and 2.45% cutoffs were selected based on the ROC curves for PB and BM samples, respectively.

**Conclusions** Quantification of large B-cells in PB and BM samples by FC is reliable and analytical performances met the acceptance criteria. Assessment of performances of different instruments and protocols is warranted.

## Introduction

Increasing interest in canine lymphoma by the scientific literature has been observed in the recent years for two reasons. The first is the high prevalence in dogs, and the second is the possible identification of the dog as spontaneous animal model to study lymphoma in humans (1-4).

However, diagnostic, staging and treatment protocols are not standardized in veterinary medicine, thus preventing the comparison of results from different studies and reducing the scientific value of the canine model. Indeed, as innovative and more sensitive staging methods are introduced, dogs may be reclassified as having a higher disease staging (5).

In this context, peripheral blood (PB) and bone marrow (BM) infiltration have been assessed alternatively by cytology (5-8), flow cytometry (FC) (9-12) or PCR for antigen receptor rearrangements (PARR) (13), but only FC has been demonstrated to have a prognostic relevance in dogs with large B-cell lymphoma (LBCL) (11). PB and BM infiltration were quantified as the percentage of large B-cells on the total CD45-positive cells. Further, BM infiltration was negatively correlated with time to progression and lymphoma specific survival and a cut-off of 3% infiltration was the best value to discriminate between two prognostic groups. Despite its efficacy to discriminate dogs with different prognosis, analytical performances of this FC strategy have never been assessed.

The aim of the present study was to explore the applicability of FC for the evaluation of PB and BM infiltration in dogs with LBCL. In particular, we aim to: 1) assess analytical sensitivity, precision and accuracy; 2) select a cut-off percentage of large B-cells suitable to safely discriminate between infiltrated and non-infiltrated PB and BM samples.

## Materials and methods

Samples included in the study were sent by private veterinarians to the Laboratory of the Department of Veterinary Sciences (University of Turin, Grugliasco, Turin) for diagnostic purposes or routine haematology analysis, and analyzed within 24 hours from sampling. All dogs were privately owned and sampled for

diagnostic purposes with the informed consent of owners. Thus, a formal approval of the Institution Committee for Animal Care of the University of Turin was not necessary.

#### *Flow cytometry*

Sample processing for FC was performed as previously described (14). Lymph node aspirates (LN) were collected in RPMI 1640 tubes, whereas PB and BM samples were collected in EDTA tubes. Prior to labelling, all samples were counted via an automated haematology analyzer (ADVIA 120, Siemens Healthcare Diagnostics, Milan, Italy) to assess cellularity and underwent RBC lysis with an erythrocytes lysis buffer containing 8% ammonium chloride. After washing, cells were resuspended in RPMI 1640 medium, containing 5% fetal bovine serum. For surface marker labelling,  $1 \times 10^6$  cells/tube were incubated with different combinations of the following antibodies: CD5-FITC (clone YKIX322.3), CD3-FITC (clone CA17.2A12), CD4-FITC (clone YKIX302.9), CD8-PE (clone YCATE55.9), CD21-PE (clone CA2.1D6), cyCD79b-FITC (clone AT107), CD45-AlexaFluor647 (clone YKIX716.13). Isotype-matched controls were included for each labelling. All antibodies were previously titrated to determine the best working dilution. All antibodies were from AbD Serotec (Oxford, UK). After incubation of 20 minutes at 4°C, samples were washed twice in RPMI 1640 and finally resuspended in 500 µl PBS for acquisition.

Samples were acquired with a BD Accuri C6 (Becton Dickinson, San José, CA, USA). For each sample, a minimum of 10,000 cells was acquired, with an acquisition rate of less than 2,000 events/second. Analyses were performed with a specific software (CFlow Plus, Becton Dickinson, San José, CA, USA) by one operator (FR). Cells were visualized at first in a FSC vs SCC morphological dot plot, and a gate (R1) was set to exclude platelets and debris. R1 cells were then visualized based on FSC and CD45 expression. Further analyses were performed including only R1 CD45-positive cells.

The diagnosis of LBCL was achieved when LN cytological findings were compatible with high-grade B-cell lymphoma according to updated Kiel classification scheme (15) and more than 80% of CD45-positive cells in the LN were large-sized (FSC-H > 2,000,000) and co-expressing CD21. To assess PB and BM infiltration,

CD45-positive R1 cells were visualized based on FSC and CD21 expression: infiltration was defined as the percentage of large CD21-positive cells out of total CD45 cells (11) (Figure 1).

#### *Analytical validation*

Dilution experiments were performed by adding LN neoplastic cells from dogs with LBCL to PB and BM samples from one healthy dog and one dog with a disease different from lymphoma (mast cell tumour), respectively. A single dilution experiment for each matrix (PB and BM) was performed. Dilution volumes were calculated in order to have neoplastic cells accounting for 50%, 25%, 10%, 7%, 5%, 3%, 2%, 1%, 0,5%, 0,4% and 0,3% out of total CD45-positive cells. To this aim, a multi-step mathematical calculation was done, including the following parameters: expected large B-cells percentage, LN and PB/BM cellularity assessed by the haematology analyser, percentage of large B-cells and of other non-neoplastic cells in the LN and PB/BM sample assessed via FC, total number of cells/tube desired. Finally, PBS 1x was added to each tube, to fill to a final volume of 100 µl. All samples were acquired immediately after preparation. Agreement between expected and obtained percentages was evaluated by Passing-Bablok regression analysis (Analyse-it, Analyse-it Software Ltd, Leeds, UK).

Negative controls (i.e. PB and BM samples prior to neoplastic cells addition), 1%, 3% and 10% samples were acquired ten-times to assess intra-assay precision as measured by the Coefficient of Variation (CV).

Sensitivity was defined as the percentage of neoplastic cells to be added to exceed the Limit Of Detection (LOD) in negative controls. The LOD was defined as mean + 3 Standard Deviation (SD) of all negative controls included in the present study (16-17).

#### *Diagnostic validation*

Two ROC curves were drawn with SPSS Statistics for Windows 17.0 (SPSS Inc, Chicago, USA), to select the large B-cells cut-off value most suitable to discriminate between infiltrated and non-infiltrated PB and BM samples. PARR-positive PB and BM samples from dogs with LBCL were regarded as truly infiltrated samples, whereas PARR-negative PB and BM samples from healthy dogs or dogs with diseases other than LBCL were regarded as truly negative samples. PARR analysis was performed according to already published protocols

(18) on LN, PB and BM samples stored at -20°C until the analysis day. The cut-offs were selected based on the best compromise between sensitivity and specificity in PB and BM samples, respectively.

## Results

### *Analytical validation*

Results from dilution experiments are listed in Table 1. Passing-Bablok regression analysis revealed an optimal agreement between expected and obtained percentages. In particular, no significant error was detected in PB dilutions (intercept 0.00, 95% CI 0.00-0.00; slope 1.00, 95% CI 0.93-1.05), whereas a slight proportional error was detected in BM dilutions (intercept 0.00, 95% CI 0.00-0.00; slope 0.89, 95% CI 0.80-0.95) (Figure 2).

Intra-assay precision parameters are listed in Table 2.

LOD was 0.470 and 1.125 in the PB and BM negative controls, respectively. A minimum of 0.5% and 2% large B-cells had to be added to PB and BM samples to exceed these values, respectively (Table 1). These values were therefore considered the lower detection limit for PB and BM samples, respectively.

### *Diagnostic validation*

Overall, 50 samples were used to draw each ROC curve, including 25 positive and 25 negative samples. PB negative samples included: 7 healthy dogs, 4 dogs with Leishmaniasis, 2 dogs with atopic dermatitis, 2 dogs with pyoderma, 2 dogs with T-cell lymphoma, 2 dogs with periodontitis, and 1 dog each with disseminated histiocytic sarcoma, Evans syndrome, immune-mediated thrombocytopenia, mast cell tumour, melanoma and osteosarcoma. Mean large B-cell percentage in the negative samples was  $0.109 \pm 0.120\%$  (median 0.08%, range 0.01-0.6%); mean large B-cell percentage in the positive samples was  $10.550 \pm 18.765\%$  (median 3.13%, range 0.2-83.6%). The ROC curve identified a 0.41% large B-cells percentage as the best cut-off to discriminate between positive and negative PB samples, with a 96% sensitivity and a 96% specificity. This value was below the lower detection limit. Among values exceeding the lower detection

limit, 0.56% large B-cells was the one associated with the best compromise between sensitivity (92%) and specificity (96%).

BM negative samples included: 7 dogs with regenerative anaemia, 7 dogs with T-cell lymphoma, 4 dogs with Leishmaniasis, 2 dogs with Evans syndrome and 1 dog each with disseminated histiocytic sarcoma, immune-mediated neutropenia, lymph node reactive hyperplasia of unknown origin, mast cell tumour, multiple myeloma. The mean percentage of large B-cells in the negative samples was  $0.340 \pm 0.267\%$  (median 0.3%, range 0.1-1.43%); the mean percentage of large B-cells in the positive samples was  $11.730 \pm 18.031\%$  (median 3.9%, range 0.45-73.6%). The ROC curve identified a 0.77% large B-cells percentage as the best cut-off to discriminate between positive and negative BM samples, with a 96% sensitivity and a 96% specificity. This value was below the lower detection limit. Among values exceeding the lower detection limit, 2.45% large B-cells was the one associated with the best compromise between sensitivity (68%) and specificity (100%).

## Discussion

Different techniques have been used in the published literature to assess PB and BM infiltration in dogs with lymphoma. However, only BM infiltration quantified by FC has proved to be prognostically relevant (11). The present study describes the analytical performances of the FC strategy routinely used in veterinary medicine. In addition, we identify the percentage cut-offs of large B-cells best discriminating between infiltrated and non-infiltrated samples.

Based on our results, FC accurately quantifies neoplastic large B-cells in canine PB and BM samples. Indeed, only a slight proportional error in BM dilutions was found. Although causes for this slight error are not neat to the authors, this may be associated to pre-analytical factors, such as manual dilutions preparation, or to the presence of stromal or adipose cells either in the LN or in the BM sample that were counted by the haematology analyzer but were not included in the FC gates. However, this proportional error has only a tenuous clinical relevance, since it affects only high infiltration values, which are uncommon in canine LBCL (11,12) and far from the prognostically relevant cut-off of 3% (11).



We also assessed analytical precision of our FC strategy, by calculating the CVs for negative controls and for three different infiltration levels. Different methods have been proposed in the published literature to assess whether the analytical imprecision of a laboratory test may be considered acceptable, correlating analytical CV to the Total Error Allowable (TEA), that is the total amount of error medically, administratively or legally acceptable (19). The calculation of TEA is based on biological variations. To date, there are no data concerning biological variations in the percentage of large B-cells in canine PB or BM samples: thus, it is not possible to evaluate the CVs obtained by the present study with traditional methods. Still, a few considerations can be done. First, because only a 3% BM infiltration is considered clinically relevant (11), and the CVs obtained for this percentage in the present study are low, they might be tentatively considered acceptable. Second, high CV values were obtained only for negative controls, but still, large B-cells percentages in the negative controls were always much lower than 3%, thus clinical decisions would not be affected. However, further studies are needed, including repeated samples from healthy dogs and from dogs of different breeds, to assess the individual and the inter-breed variability and define the most appropriate TEA.

We identified a sensitivity of 0.5% and 2% for our FC strategy applied to PB and BM samples, respectively.

The sensitivity of FC is influenced by many variables. First, it increases in parallel with the number of evaluated parameters and antigen expression. Unfortunately, this is hard to achieve in veterinary medicine because of the paucity of validated antibodies cross-reacting with canine antigens. In particular, canine B-cell markers that may be investigated via FC are mostly represented by intracellular molecules or cytoplasmic portion of intra-membrane proteins: permeabilization procedures are then required, which are time-consuming, enhance costs and are therefore of scarce applicability to clinical diagnostic assays.

Validation of new antibodies is warranted, to get a more detailed finger-print of neoplastic cells and to improve sensitivity in staging procedures. Second, sensitivity varies with the number of acquired events. In the present study, we acquired 10,000 cells/tube in order to follow as closely as possible the procedures described in literature and applied by most of the veterinary diagnostic laboratories. However, we support the opportunity of acquiring a higher number of events (up to 100,000) to analyze clinical specimens where only a low number of neoplastic cells is detected, in order to improve sensitivity.

Beside analytical validation, we also managed to detect a large B-cells cut-off suitable to safely discriminate between infiltrated and not-infiltrated PB and BM samples. In the absence of an officially recognized gold standard, PARR-positive samples were considered as truly infiltrated because PARR is more sensitive than FC in detecting infiltrated samples at diagnosis (18). Unfortunately, false negative PARR results have also been reported in dogs with lymphoma (20,21): thus, PB or BM infiltration should not be excluded based on PARR-negative results and these samples could not be considered as truly not-infiltrated. That being so, we only considered PARR-negative samples from healthy dogs or dogs with other diseases as truly negative samples.

Several studies in human medicine described the use of FC to stage B-cell lymphomas. High agreement between FC and BM biopsy (BMB) results has been reported, although also FC-positive BMB-negative and FC-negative BMB-positive cases might occur (22-24). However, differently from the present study, the FC strategy used in human medicine is based on the calculation of  $\kappa/\lambda$  ratio (light chain restriction).

Unfortunately, this approach is ineffective in the canine species, since even healthy dogs have 90%  $\lambda$  chain and a restricted ratio (25). In addition, most commonly applied diagnostic criteria vary between human and canine lymphomas. Indeed, histopathological evaluation is mandatory under official guidelines in human medicine (26), whereas it is not commonly performed in dogs when a cytological diagnosis of high-grade lymphoma has been obtained, as documented by a recent survey among veterinary oncologists (27). Given these circumstances, in the present study the diagnosis of LBCL was made based only on neoplastic cells cytological appearance and FC phenotype, even if different lymphoma subtypes may have been included.

The main pitfall of the present study is that the research protocol has been performed by one operator, with one single flow cytometer associated to a specific software. Thus, our results may not be expandable to other laboratories, since slight operator-to-operator or machine-to-machine variability is possible (28). However, this is the first study doing this approach representing a milestone for other FC facility to assess analytical and diagnostic performances.

In conclusion, the present study describes a FC strategy to quantify PB and BM neoplastic cells infiltration in dogs with LBCL and analytical and diagnostic performances met the acceptance criteria. Validation of

laboratory methods to stage canine lymphoma is relevant not only from a veterinary point of view, but also from a comparative perspective, since the dog is increasingly regarded as a valid animal model to study human lymphoma (1-4).

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**Table 1:** Percentages of large B-cells obtained diluting lymph nodal neoplastic cells from a dog with large B-cell lymphoma into negative peripheral blood and bone marrow controls at different concentrations

Expected large B-cell concentration (%)	Obtained large B-cell concentration (%)	
	Peripheral blood	Bone marrow
Negative control	0.230 (CV=50.413%)*	0.420 (CV=21.879%)*
0.3	0.4	Nt
0.4	0.4	0.5
0.5	0.6	0.8
1	1.030 (CV=9.211%)*	0.950 (CV=10.230%)*
2	1.6	1.5
3	2.900 (CV=3.635%)*	2.890 (CV=7.194%)*
5	5.3	3.9
7	7.1	6
10	9.500 (CV=3.746%)*	9.870 (CV=2.484%)*
25	27.8	23.4
50	49.9	44.7

\*=mean value of ten repeated measurements; Nt= not tested

**Table 2:** Intra-assay precision of large B-cells quantification in dilutions of lymph node neoplastic cells from a dog with large B-cell lymphoma into negative peripheral blood and bone marrow controls

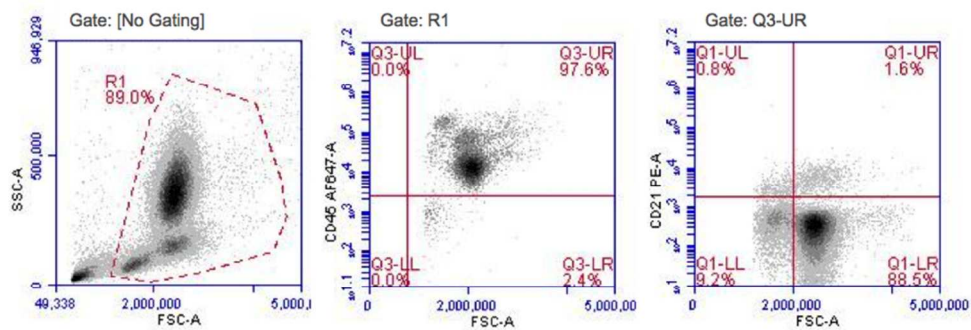
Expected large B-cell concentration (%)	PB dilutions		BM dilutions	
	mean±SD	CV (%)	mean±SD	CV
Negative control	0.230±0.116	50.413	0.420±0.092	21.879
1	1.030±0.095	9.211	0.950±0.097	10.230
3	2.900±0.105	3.635	2.890±0.208	7.194
10	9.500±0.356	3.746	9.870±0.245	2.484

PB= peripheral blood; BM= bone marrow; SD=standard deviation; CV= coefficient of variation

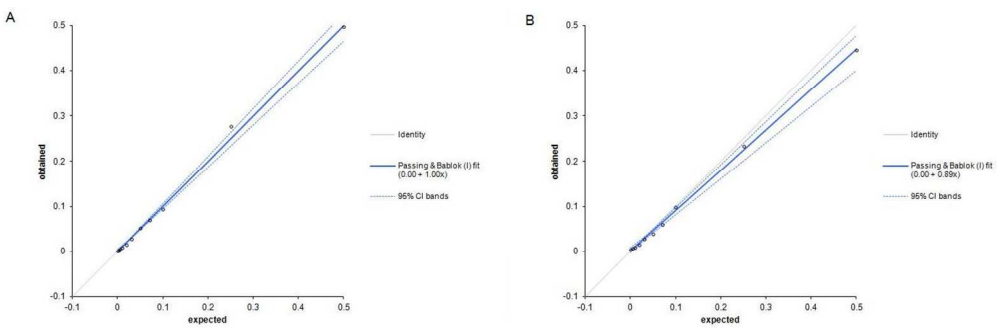
**Figure 1:** scatter plots representing our gating strategy. Cells were visualised at first in a FSC vs SCC morphological dot plot and a gate (R1) was set to exclude platelets and debris. R1 cells were then visualized based on FSC and CD45 expression. Finally, CD45-positive R1 cells (Q3-UR) were visualized based on FSC and CD21 expression. Sample infiltration was defined as the percentage of large CD21-positive cells out of total CD45-positive cells.

**Figure 2:** results from Passing-Bablok regression analyses performed to assess the agreement between expected and obtained large B-cells percentages in dilution experiments. **A:** peripheral blood dilution experiments; no error was detected. **B:** bone marrow dilution experiments; a slight proportional error was found.





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